DMD #24745

Species differences in blood-brain barrier transport of three PET radioligands with emphasis on P-glycoprotein transport

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1

DMD #24745

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D. Nonstandard abbreviations:

5HT receptor	serotonin receptor
CsA	Cyclosporin A
f_p	plasma protein unbound fraction
IC50	Concentration of inhibitor producing 50% of maximal inhibition

DMD #24745

K_p	brain-to-plasma concentration ratio
$K_{p,increase}$	increase in Kp after CsA administration
NK_1-receptor	Neurokinin 1 receptor
PET	Positron Emission Tomography
P-gp	P-glycoprotein
ROI	region of interest
R_{buffer}	radioactivity concentration in buffer
R_{plasma}	radioactivity concentration in plasma
SUV	standardized uptake value
$SUV_{increase}$	increase in SUV after CsA administration

DMD #24745

Abstract

Species differences occur in the brain concentrations of drugs, but the reasons for these differences are not yet apparent. This study was designed to compare brain uptake of three radiolabelled P-glycoprotein (P-gp) substrates across species using Positron Emission Tomography (PET). Brain concentrations and brain-to-plasma ratios were compared; [^{11}C]verapamil in rats, guinea pigs and monkeys, [^{11}C]GR205171 in rats, guinea pigs, monkeys and humans, and [^{18}F]altanserin in rats, minipigs and humans. The fraction of the unbound radioligand in plasma was studied along with its metabolism. The effect of P-gp inhibition was investigated by administering cyclosporin A (CsA). Pronounced species differences were found in the brain and brain-to-plasma concentrations of [^{11}C]verapamil, [^{11}C]GR205171 and [^{18}F]altanserin with higher brain distribution in humans, monkeys and minipigs than in rats and guinea pigs. For example, the brain-to-plasma ratio of [^{11}C]GR205171 was almost 9-fold higher in humans compared to rats. The species differences were still present after P-gp inhibition although the increase in brain concentrations after P-gp inhibition was somewhat greater in rats than in the other species. Differences in plasma protein binding and metabolism did not explain the species-related differences. The findings are important for interpretation of brain drug delivery when extrapolating preclinical data to humans. Compounds found to be P-gp substrates in rodents are likely to also be substrates in higher species but sufficient blood-brain barrier permeability may be retained in humans to allow the compound to act at intracerebral targets.

DMD #24745

Introduction

Species differences in receptor and enzyme systems are well known, but very little is known about species differences in the transport of drug molecules across the blood-brain barrier. Transport across this barrier occurs by passive diffusion either alone or in combination with active influx or efflux. P-glycoprotein (P-gp; ABCB1; MDR1) is an ATP-dependent efflux transporter that carries a wide range of drug molecules at the blood-brain barrier as well as in other organs such as the liver, kidney and gastrointestinal tract. It has a profound influence on brain concentrations of its substrates. Due to its non-invasiveness, Positron Emission Tomography (PET) is one of the few methods that allows for direct *in vivo* comparisons between preclincal species and humans regarding drug interactions with P-gp.

[11C]verapamil is the most frequently used PET radioligand for P-gp studies (Hendrikse et al., 1999; Bart et al., 2003; Sasongko et al., 2005; Lee et al., 2006; Syvänen et al., 2006). Other examples include [11C]carazolol, [18F]fluorocarazolol, [11C]carvedilol, [11C]GR218231, [11C]loperamide and [11C]desmethyl loperamide (Doze et al., 2000; Bart et al., 2005; de Vries et al., 2005; Lazarova et al., 2008; Zoghbi et al., 2008). Some radioligands shown to be P-gp substrates in rodents are nonetheless taken up by the brain in primates, indicating that there may be species differences in P-gp function. For example, Liow *et al.* have shown that the 5HT$_{1A}$ receptor antagonist [11C]RWAY is a P-gp substrate in the rat and mouse and displays a low brain uptake in these species (Liow et al., 2007). However, uptake of [11C]RWAY in human and monkey brains is relatively high (Yasuno et al., 2006; Zhang et al., 2007). Similarly, while uptake of the chemically

5

related 5HT$_{1A}$ receptor antagonist [^{18}F]MPPF by the human brain is relatively high, the

molecule has been shown to be a P-gp substrate in rats (Passchier et al., 2000a; Passchier

et al., 2000b). Further, GR205171, an NK$_1$ receptor antagonist, is a P-gp substrate in

rodents (Rupniak et al., 2003) but uptake of radioactively labelled [^{11}C]GR205171 is high

in human and monkey brains (Bergström et al., 2000; Furmark et al., 2005; Michelgard et

al., 2007). Uptake of [^{18}F]altanserin, a 5HT$_{2A}$ receptor antagonist, in human, baboon and

pig brains is also relatively high (Biver et al., 1994; Staley et al., 2001). However, initial

ex vivo studies of [^{18}F]altanserin in rats have shown relatively low brain concentrations

(unpublished data, NRU Copenhagen, Denmark).

One explanation for the species differences in brain concentrations observed with these

radioligands could be the existence of a species difference in P-gp function, despite the

high degree of homology in the amino acid content of P-gp in different species (Table 1).

Several *in vitro* studies have shown that the transport capacity of P-gp substrates differs

among cell lines transfected with P-gp from different species, indicating a species

difference in substrate recognition (Yamazaki et al., 2001; Ohe et al., 2003; Katoh et al.,

2006; Xia et al., 2006; Baltes et al., 2007). Besides differences in substrate affinity,

differences in P-gp expression could also result in differences in P-gp transport capacity.

In vivo evidence for species differences in P-gp function has so far only been reported by

one group, who compared rats and guinea pigs and found that higher concentrations of

the P-gp inhibitor GF-120918 were needed in guinea pigs than in rats to achieve the same

increase in brain concentrations of the P-gp substrate SB-487946 (Cutler et al., 2006). In

contrast, when Hsiao *et al*. compared the increase of [^{11}C]verapamil in the human and rat

brain after treatment with low doses of the potent *in vivo* P-gp inhibitor cyclosporin A

(CsA) they did not observe any species differences (Hsiao et al., 2006). Other

explanations could be species differences in blood-brain barrier transport that are not

related to P-gp function, but rather to other causes, e.g. tightness of the endothelial cell

tight junctions or expression of other active efflux transporters. Differences in intrabrain

distribution due to differences in lipophilic content of brain tissue, and in the degree of

plasma protein binding, metabolism and elimination from plasma could also lead to

different levels of brain uptake among species.

The *in vivo* brain uptake of [11C]verapamil, [11C]GR205171and of [18F]altanserin in

several species was investigated in this paper. Interspecies comparisons of brain

concentrations and brain-to-plasma concentration ratios, fraction of unbound radioligand

in plasma and radioligand metabolism were carried out. The effect of P-gp was studied

by administering CsA to a subgroup of animals.

DMD #24745

Materials and Methods

General

The radioligands [11C]verapamil, [11C]GR205171 and [18F]altanserin, Figure 1, were synthesised as previously reported (Lemaire et al., 1991; Bergström et al., 2000; Syvänen et al., 2006). All chemicals were obtained commercially and were of reagent grade or better. The P-gp inhibitor CsA (Sandimmun®, Novartis, Basel, Switzerland) was diluted to the desired concentration in saline. All animal experiments were approved by the Uppsala Animal Ethics Committee (C117/4 and C 153/4) and the Danish Animal Research Inspectorate (Journal number 2003/561-745). The clinical data used in this paper were acquired from clinical studies in healthy volunteers (Syvänen, 2003; Liptrot et al., 2004) and were performed in accordance with the Helsinki declaration. A summary of the number of subjects for each radioligand and species is given in Table 2.

Anaesthesia and surgical procedures

Before the experiments, rats and guinea pigs were housed in ventilated cabinets at a room temperature of 20±2°C, were maintained on a 12 h light: 12 h dark cycle and were given unlimited access to food and water. Gaseous anaesthesia with isoflurane 2.6% (Baxter Medical AB, Kista, Sweden) in a mixture of oxygen and medical air (40%/60%, AGA Gas AB, Sundbyberg, Sweden) was used for the rat and guinea pig studies with [11C]GR205171 and [11C]verapamil, while hyponorm/midazolam (VetaPharma Ltd., Leeds, United Kingdom/Hameln Pharmaceuticals, Hameln, Germany) was used for the studies with[18F]altanserin. After anaesthesia, a femoral artery cannula was inserted for blood sampling and an intravenous catheter was inserted into the left femoral vein in

8

guinea pigs and the tail vein in rats for radioligand and CsA administration. The

[^{11}C]GR205171 and [^{11}C]verapamil uptake in rats and guinea pigs was studied

dynamically with PET, while the brain uptake of [^{18}F]altanserin was measured after

euthanization of the animals (*ex vivo* studies).

The minipigs used for the [^{18}F]altanserin studies were initially anaesthetized with 0.15

ml/kg i.m. of midazolam (Hameln Pharmaceuticals, Hameln, Germany) plus Zoletil 50

vet. (Virbac Animal Health, Carros, France). One ampoule of Zoletil 50 vet (containing

125 mg each of zolazepam and tiletamine) was dissolved in 8 ml midazolam solution (1

mg/ml). The anaesthesia was maintained with i.v. propofol (1 ml/kg/h) (Fresenius Kabi

AB, Uppsala, Sweden). The minipigs were intubated endotracheally and ventilated

during the entire experiment. During the scan, they were placed on a heating carpet and

covered to maintain body temperature. Catheters were surgically inserted in the femoral

vein for radioligand and CsA administration, and into the femoral artery for blood

sampling. Body temperature and physiological functions (blood pressure, blood

oxygenation and heart rate) were monitored continuously and haematocrit, blood glucose

and acid–base parameters (pH, pCO_2, pO_2, H_2CO_3 and O_2 saturation) were measured in

full blood samples on an ABL system (625, Radiometer, Copenhagen, Denmark) at

regular intervals. Deviations from normal values were corrected appropriately.

The cynomolgus monkeys were transported to the investigation site at Uppsala Imanet on

the morning of the experiment. Venous catheters were inserted in both hind legs and

propofol (50 mg, Lipuro®, B.Brown Medical AB, Bromma, Sweden) was administered

to induce anaesthesia. The catheters were also used for administration of the radioligands

and Ringer-Acetate (2 ml/kg/h, Fresenius Kabi AB, Uppsala, Sweden). The animals were

kept anaesthetised with 1.3 - 2.5% sevoflurane (Abbott Scandinavia AB, Solna, Sweden)

via tracheal intubation during the PET scan. A venous catheter in the tail was used for

blood sampling. One monkey had two PET scans and another had three. The PET scan

was started when the radioligand was administered. Data from an earlier study with

[11C]GR205171 in rhesus monkeys were also included in this study (Syvänen et al.,

2007).

Blood (0.2 ml) was sampled 0.5, 1, 3, 5, 10, 15, 20, 30, 45 and 60 min after

administration of all compounds for all species and the radioactivity concentration in

blood and plasma was measured in a well counter that was cross-calibrated with the PET

system. Additional blood samples (0.3 ml) were obtained at 0, 15, 30 and 60 min for

measurement of CsA concentrations in whole blood using an immunoenzymatic assay

(CEDIA, Microgenics, Ekerö, Sweden) with a limit of detection of 25 ng/mL. Three to 5

blood samples (1-2 ml) were obtained for metabolite analysis in minipigs, monkeys and

humans during the scan.

 PET scanners

In rodents, a microPET R4 scanner (Concorde Microsystems, Knoxville, TN, USA) was

used for [11C]GR205171 and [11C]verapamil studies. In minipigs, a combined PET/CT

Discovery LS Scanner (General Electrics, Milwaukee, WI, USA) was used for

[18F]altanserin studies and, in monkeys, a Hamamatsu SHR-7700 tomograph

(Hamamatsu Photonics, Hamamatsu, Japan) was used for [11C]GR205171 and

[11C]verapamil studies. The human [11C]GR205171 studies were performed using an

ECAT EXACT HR+ scanner (Siemens/CTI, Knoxville, TN, USA) and the [18F]altanserin

studies used a GE Advance scanner (General Electrics, Milwaukee, WI, USA).

Transmission scans, used for correction of attenuation, were run prior to the emission

scans.

Studies with [11C]verapamil

Six rats (mean weight 412 ± 45 g) received [11C]verapamil as a bolus injection of 26 ± 11

MBq. Three of them received a bolus dose of 22.5 mg/kg CsA followed by a constant

infusion of 7.5 mg/kg/h, starting 20-25 min prior to administration of the radioligand. The

same protocol was used for six guinea pigs (mean weight 437 ± 48 g) at a [11C]verapamil

dose of 15 ± 5 MBq. One female cynomolgus monkey (weight 3.8 kg) received a bolus

injection of [11C]verapamil 68 MBq. Four and half hours later, a 15 mg/kg CsA bolus

was followed by a constant infusion of 5 mg/kg/h, started 20-25 min prior to the second

[11C]verapamil bolus of 74 MBq. Regions of interest (ROIs) were drawn over the

transaxial brain images in the region of the cerebellum.

Studies with [11C]GR205171

Nine rats (mean weight 400 ± 26 g) received a bolus injection of 26 ± 13 MBq

[11C]GR205171. Three of them received a bolus dose of 22.5 mg/kg CsA followed by a

constant infusion of 7.5 mg/kg/h, while three others were administered a bolus dose of

7.5 mg/kg CsA followed by a constant infusion of 2.5 mg/kg/h, starting 20-25 min prior

to administration of the radioligand. Six guinea pigs (mean weight 488 ± 58 g) received a bolus injection of 27 ± 8 MBq [^{11}C]GR205171. Three of them received a bolus dose of 22.5 mg/kg CsA followed by a constant infusion of 7.5 mg/kg/h, starting 20-25 min prior to the radioligand administration. The first of two female cynomolgus monkeys (weights 6.4 and 3.8 kg) received [^{11}C]GR205171 as a single bolus of 95 MBq, and 4.5 hours later received a bolus dose of 15 mg/kg CsA and a constant infusion of 5 mg/kg, 20-25 min prior to a second bolus dose of 112 MBq [^{11}C]GR205171. The second animal received one single bolus dose of 78 MBq [^{11}C]GR205171. ROIs were drawn over the transaxial brain in the region of the cerebellum where the distribution of NK_1 receptors is negligible (Bergström et al., 2000; Haneda et al., 2007). In the monkeys, ROIs were also drawn over the striatum where NK_1 receptors are abundant.

Studies with [^{18}F]altanserin

Nine rats (mean weight 371 ± 70 g) received 8 ± 4 MBq [^{18}F]altanserin as a bolus injection. Four of them received a 22.5 mg/kg bolus of CsA followed by a constant infusion of 7.5 mg/kg/h, starting 20-25 min prior to the administration of radioligand. Six Göttingen minipigs (mean weight 31 ± 8 kg) received a bolus injection of 197 ± 154 MBq [^{18}F]altanserin. One of them received a bolus of 22.5 mg/kg of CsA followed by a constant infusion of 7.5 mg/kg/h starting 20-25 min prior to the radioligand administration. In rat studies, the brain was dissected at 60 min and the radioactivity was measured in the cerebellum, where the distribution of $5HT_{2A}$ receptors is negligible, and frontal cortex, in which the density of $5HT_{2A}$ receptors is high (Biver et al., 1994). In

minipig studies, ROIs were automatically applied in the region of the cerebellum and the

frontal cortex using a minipig brain atlas (Watanabe et al., 2001).

Plasma protein binding

The extent of plasma protein binding was determined using equilibrium dialysis. Plasma

samples were obtained, 1-2 MBq of the radioligand was added to each sample, and 500

µL of plasma was then transferred to a dialysis chamber with a semipermeable membrane

separating the other chamber containing 500 µL of PBS buffer. After the chambers had

been shaken in a 37°C water bath for 2 hours, a sample from each chamber was removed

and the radioactivity concentrations were measured. The plasma protein unbound

fraction, f_p, was calculated as:

$$f_p = \frac{R_{buffer}}{R_{plasma}} * 100 \tag{1}$$

R_{buffer} and R_{plasma} are the radioactivity concentrations in the buffer and plasma chambers,

respectively. All values are based on $n \geq 3$ unless otherwise stated, and are reported as

means and standard deviations. A two-tailed t-test was used to test whether the difference

in plasma protein binding between plasma containing and not containing CsA was

statistically significant.

Metabolism

The metabolism of [^{11}C]verapamil and [^{11}C]GR205171 was evaluated in rat and guinea

pig plasma. The metabolism of [^{18}F]altanserin in rats has been studied previously (Tan et

al., 1999). The metabolism of [^{11}C]verapamil and [^{11}C]GR205171 in monkey plasma and

the metabolism of [^{18}F]altanserin in minipigs was performed in parallel with the imaging

studies. The methods for separating and analysing parent and metabolite fractions of

[^{11}C]verapamil, [^{11}C]GR205171 and [^{18}F]altanserin have been published previously

(Bergström et al., 2000; Pinborg et al., 2003; Syvänen et al., 2006).

Data analysis

Radioactivity measurements in plasma were corrected for metabolism before analysis.

The time-activity profiles in the brain and plasma were expressed using the Standardized

Uptake Values (SUV; normalised concentrations) to enable comparison between scans.

$$SUV = \frac{Measured\ Radioactivity\ in\ tissue}{Injected\ Radioactivity / Body\ Weight} \qquad (2)$$

To account for individual and species differences in the plasma concentrations, which are

the driving force for the brain concentrations, the brain-to-plasma ratio K_p was calculated

using the SUV in plasma and brain.

$$K_p = \frac{SUV\ brain\ region}{SUV\ plasma} \qquad (3)$$

SUV in brain and plasma and K_p were calculated over the scanning time, one value for

each PET time frame. Average SUV and K_p values for each animal were also determined

from calculations of SUV and K_p every 5 min between 0 and 60 min. All values are

14

DMD #24745

reported as means and standard deviations. The effect of P-gp inhibition was calculated

by comparing SUV or K_p in CsA-untreated and -treated animals.

$$SUV_{increase} = \frac{SUV_{average\ 0-60\ min}\ with\ CsA}{SUV_{average\ 0-60\ min}\ baseline} \qquad (4)$$

$$K_{p,increase} = \frac{K_{p\ average\ 0-60\ min}\ with\ CsA}{K_{p\ average\ 0-60\ min}\ baseline} \qquad (5)$$

SUV_{increase} and K_{p,increase} were calculated from the average values for SUV and K_p.

K_{p,increase} takes into account CsA-induced differences in the plasma pharmacokinetics and

may therefore be a more reliable parameter than SUV_{increase} for assessing the change in

CsA-induced P-gp inhibition. A one-tailed t-test was used to see if the increase was

statistically significant.

DMD #24745

Results

Species differences in radioligand brain and plasma concentrations

Distribution to the brain of the three studied radioligands [^{11}C]verapamil, ^{11}C]GR205171 and [^{18}F]altanserin were clearly higher in humans, monkeys and minipigs than in rats and guinea pigs, as described with brain concentrations, SUV, and the brain-to-plasma concentration ratios, K_p (Figures 2 – 4, Table 3). For [^{11}C]verapamil, the brain distribution was highest in monkeys, followed by rats and guinea pigs with K_p-vales of 4.6 to 1.1 and 0.65, respectively (Table 3). Monkeys had a 4-fold higher brain distribution ratio (K_p) than rats. The same trend was observed with [^{11}C]GR205171 and [^{18}F]altanserin, where monkeys and minipigs showed higher brain uptake than rodents (Table 3). PET images with [^{11}C]GR205171 in monkey, guinea pig and rat are shown in Figure 5. Humans had a [^{11}C]GR205171 K_p ratio of 30, while rats had a ratio of 3.4, thus an 8.6-fold higher brain distribution in humans than in rats, and 3-fold higher in humans than in monkeys. The K_p of [^{18}F]altanserin was 4.5-fold higher in humans than in rats.

The metabolite corrected plasma SUV values after [^{11}C]verapamil administration, describing the drug concentrations in plasma in relation to dose administered (Equation 2), were highest in guinea pigs, followed by rats and monkeys. For [^{11}C]GR205171 plasma SUVs were highest in monkeys, followed by guinea pigs and then humans and rats. Plasma SUV values of [^{18}F]altanserin were highest in rats, followed by humans and minipigs. Thus, in contrast to the brain concentrations of the three radioligands, no tendency towards lower concentrations in the rodents than in the higher species was observed.

Effect of CsA induced P-gp inhibition

The potent P-gp inhibitor CsA was administered to a subgroup of the animals (not to humans). Since plasma concentrations are the driving force for brain concentrations, it is more relevant to compare the K_p than the SUV values with and without CsA treatment to obtain information on how much of the increase is due to P-gp inhibition. K_p was increased in all species after CsA administration, apart from the K_p of [^{18}F]altanserin in the minipig (Table 4). The increase was highest in rats for all three radioligands. From a clinical point of view, where the actual brain concentrations are of interest for a given dose, the increase in SUV may also be of interest. Both, $K_{p,increase}$ and $SUV_{increase}$ are therefore shown in Table 4. PET images with and without CsA treatment for [^{11}C]GR205171 in monkey, guinea pig and rat are shown in Figure 5, for [^{11}C]verapamil in the monkey are shown in Figure 6 and [^{18}F]altanserin in the mini pig are shown in Figure 7.

The CsA concentrations influence the degree of P-gp inhibition. The mean CsA concentrations were 19.1 ± 9.3 µg/mL in guinea pigs and 11.7 ± 1.7 µg/mL in rats. Three of the [^{11}C]GR205171 administered rats received a lower dose of CsA which resulted in a mean CsA concentration of 2.2 ± 0.5 µg/mL. The two monkeys treated with CsA had somewhat different CsA concentrations despite being dosed according to weight. The monkey receiving [^{11}C]verapamil showed CsA concentrations ranging from 10 µg/mL at the beginning of the scan to 6.6 µg/mL at the end, while the monkey receiving [^{11}C]GR205171 showed CsA concentrations ranging from 20 µg/mL at the beginning of

the scan to 16 μg/mL at the end. In the [^{18}F]altanserin administered minipig the CsA

concentration continued to increase throughout the scan, ranging from 62 μg/mL at the

start to 73 μg/mL at 60 min, i.e. it was about three times higher than in rats. In spite of

this, the minipig studied did not show an increase in K_p, although the brain SUV

increased almost 2-fold (Table 4).

Metabolism of the radioligands

The metabolism of [^{11}C]verapamil in plasma was affected by CsA, which resulted in a

lower fraction of intact [^{11}C]verapamil in CsA-treated animals. In the monkey; half of the

radioactivity originated from metabolites at 24 min in CsA-untreated animal, compared to

17 min in the CsA-treated animal. CsA did not affect the metabolism of [^{11}C]GR205171

and [^{18}F]altanserin. The metabolism of [^{11}C]GR205171 was very similar in rats and

guinea pigs. After about 40 min, half of the radioactivity in plasma from these species

originated from metabolites whereas, in humans and monkeys, this point had been

reached by 20 min, thus a slower metabolism in the rodents. Plasma samples from rats 4

hours after [^{18}F]altanserin administration consisted of around 80% intact radioligand (Tan

et al., 1999). In [^{18}F]altanserin administered minipigs and humans, half of the

radioactivity in plasma originated from metabolites at around 80 and 90 min,

respectively.

The plasma protein unbound fraction

The f_p values for radioligands in the different species are shown in Table 5. The f_p for

[^{11}C]verapamil was highest in monkeys, followed by rats and guinea pigs. No species

difference in protein binding was observed for $[^{11}C]$GR205171 and $[^{18}F]$altanserin, although the f_p was very low for $[^{18}F]$altanserin making a reliable measurement difficult. A general trend was that the f_p was somewhat increased after CsA administration, but the increase was only significant in $[^{11}C]$GR205171 administered rats.

Discussion

This paper reports pronounced species differences in the brain pharmacokinetics of three PET radioligands that are P-gp substrates, both with and without CsA-induced P-gp inhibition. Rats and guinea pigs showed lower K_p ratios for the radioligands studied, than minipigs, monkeys and humans. This could be due to more P-gp present in the blood-brain barrier of rodents, or due to more efficient P-gp in these species leading to a higher P-gp mediated transport capacity. Alternatively, there could be species related differences in plasma protein binding leading to different concentrations of molecules available for transport across the blood-brain barrier. Finally, also differences in the intrabrain distribution due to differences in brain tissue properties could lead to the observed species differences.

If transport capacity would be the only explanation to the differences observed when P-gp is functioning normally, complete P-gp inhibition should result in similar brain-to-blood concentration ratios (K_p) across all species. However, the large species differences in brain concentrations were not abolished after P-gp inhibition in the present paper.

DMD #24745

Hence, our conclusion is that the differences in P-gp transport capacity alone do not explain the observed species differences.

In vitro studies have demonstrated differences in the efficacy of P-gp-mediated transport in cell lines transfected with the MDR1 gene (gene coding for P-gp) from different species. In general, most of the evaluated substrates are transported to a higher extent by mouse or rat P-gp than by human P-gp (Yamazaki et al., 2001; Ohe et al., 2003; Katoh et al., 2006; Baltes et al., 2007). However, there are exceptions. CsA, for example, was more effectively transported in cells transfected with human P-gp than in those transfected with rat or mouse P-gp (Katoh et al., 2006; Baltes et al., 2007). Another *in vitro* study showed that the IC50 of typical P-gp inhibitors differed with species and substrate (Suzuyama et al., 2007). Cutler *et al.* demonstrated *in vivo* differences between rats and guinea pigs in the IC90 of the P-gp inhibitor GF-120918 (Cutler et al., 2006). They showed that a higher plasma concentration of GF-120918 was needed in guinea pigs than in rats to achieve the same increase in brain concentrations of a P-gp substrate, SB-487946. Taken together, the *in vitro* and *in vivo* studies indicate that both transport capacity of P-gp and the IC50 values of P-gp inhibitors may differ among species. This would support the hypothesis that there are species differences in both P-gp transport and the IC50 of CsA, and may explain the observed *in vivo* species differences at both baseline and during P-gp inhibition in the present paper.

Species differences in intrabrain distribution could also be a relevant explanation for our results. The total substrate brain concentrations observed with PET will depend on the

binding potential of the substrate to brain tissue. In this instance, the term binding is

taken to include both specific binding to receptors and nonspecific binding to brain tissue

components. Brain concentrations of the radioligand will be higher in species with a

higher binding potential to brain tissue than in those with a lower binding potential.

Species differences in passive transport over the blood-brain barrier would lead to the

same outcome as differences in the intrabrain distribution since both active and passive

transport contribute to the K_p. However, it is unlikely that the physico-chemical

properties of, and tightness of tight junctions in, the blood-brain barrier itself would differ

to a large extent among species (Cserr and Bundgaard, 1984). Further, the expression of

other active transporters such as Mrp, Oat, and Oatp could also contribute to the species

differences noted.

Other possible explanations could be differences in the plasma protein binding of the

substrate. A lower level of plasma protein binding in one species would, given the same

total plasma concentration, contribute to higher brain concentrations, as more substrate

molecules would be available for transport across the blood-brain barrier. Indeed, in our

study, plasma protein binding was somewhat lower in the higher species than in rats and

guinea pigs, but the differences were not large enough to explain the species differences

in brain concentrations.

To account for species-related differences in the metabolism of the radioligands, which

could have contributed to differences in brain concentrations, the plasma concentrations

were corrected for metabolites carrying the radioactive label in the calculation of K_p in

our study. Nonetheless, the entry of radioactive metabolites into the brain could also

conceivably have affected our results, since the brain kinetics could vary among species

depending on the relative fractions of intact radioligand and radiolabelled metabolites.

This is not, however, a very likely explanation for the species differences seen in this

study since the differences appeared right from the start of the PET scans, when most of

the radioactivity originated from the intact radioligand. Previous studies focusing in more

detail on the metabolism of [11C]verapamil and [18F]altanserin in the rat has showed that

after [11C]verapamil i.v. administration most activity in the brain originates from intact

[11C]verapamil and that no lipophilic metabolites are formed during the metabolism of

[18F]altanserin (Tan et al., 1999; Luurtsema et al., 2005; Syvänen et al., 2008). Thus,

metabolism and plasma protein binding were ruled out as explanations for the species

differences.

There were some differences in the anesthesia protocols of the different experiments.

Hypnorm, which was used in the [18F]altanserin study in rat, contains fentanyl. Fentanyl

has been shown to be a P-gp substrate (Henthorn et al., 1999) and could therefore in

theory increase the uptake of [18F]altanserin by competing for P-gp substrate binding

sites. Isoflurane, which was used in the rodent [11C]verapamil and [11C]GR205171

studies, has been shown to moderately increase radioligand binding (Tsukada et al., 1999;

Elfving et al., 2003; Harada et al., 2004). Both Hypnorm and isoflurane anesthesia may

therefore reduce the measured difference in uptake between rodents and humans rather

than to be the cause of the difference. Isoflurane has been shown to decrease the cerebral

blood flow (Tsukada et al., 1999). This could indirectly change the radioligand uptake in

brain and may be a confounding factor for the results. However, based on the results

obtain by Tsukada *et al.* (Tsukada et al., 1999), the change in cerebral blood flow was too

small to explain the differences between the rats and the humans.

Although our study of [^{11}C]verapamil included only two PET-scans in monkeys, one

untreated and one receiving CsA, our results are in line with those in other publications.

For example, Lee *et al.* studied uptake of [^{11}C]verapamil after P-gp inhibition, and

reported that a 2.3-fold increase in K_p values after a 20 mg/kg infusion over 2 hours of

the P-gp inhibitor PSC833 (P-gp inhibition potency is similar to that of CsA), starting one

hour before the PET scan (Lee et al., 2006). This is to be compared with our 3.6-fold

increase.

The CsA concentrations in our study were rather similar between species, apart from in

the minipig. The guinea pigs had higher CsA concentrations than the rats. Still, the

increase in K_p was smaller in the guinea pigs than in the rats. This may underestimate the

K_p difference between guinea pigs and rats, rather than being an explanation to the

difference. The CsA-treated [^{11}C]verapamil monkey had lower CsA plasma

concentrations than the corresponding rodents, which may have resulted in relative

underestimation of $K_{p,increase}$ in the monkey.

Liow *et al.* speculated that P-gp inhibition caused a smaller increase in [^{11}C]RWAY

concentrations in the cerebellum than in $5HT_{1A}$ receptor-rich regions (Liow et al., 2007).

Our results showed a similar trend; $SUV_{increase}$ and $K_{p,increase}$ values were somewhat

smaller in the cerebellum than in the high binding regions of striatum and frontal cortex for [^{11}C]GR205171 and [^{18}F]altanserin, respectively. In line with these results, Lacán *et al.* showed in a recent paper, a somewhat larger increase in brain uptake of [^{18}F]MPPF in the 5HT$_{1A}$ receptor rich area of hippocampus compared to cerebellum after P-gp inhibition (Lacan et al., 2008). Regional differences could be attributed to heterogeneous distribution of P-gp within the brain. In the study by Lacán *et al*. P-gp protein was expressed more in cerebellum than in other brain regions, a somewhat contradictory finding to the results reported above (Lacan et al., 2008).

In the development of new drugs, *in vitro* methods of screening for P-gp-mediated transport and *in vivo* animal models are usually utilized before clinical experiments in humans. If a drug that is intended for a target inside the brain is found to be a P-gp substrate or is found in a rat model to have low brain concentrations due to it being a P-gp substrate, it might simply be discarded from further development. However, this study showed that such compounds, even though they are likely to be P-gp substrates in humans as well, could reach relatively high total brain concentrations in humans. In fact, [^{18}F]altanserin and [^{11}C]GR205171 were successfully used in humans before they were found to be P-gp substrates. The findings are important for the interpretation of brain drug delivery when extrapolating preclinical data to humans.

In conclusion, pronounced species differences were found in the brain-to-plasma concentrations of three radiolabelled molecules used in PET. It is suggested that the differences observed is a combination of species related differences in P-gp transport,

DMD #24745

potency of CsA to inhibit P-gp and possibly differences in the intra brain distribution of the molecules. Species differences should be considered when extrapolating data obtained in animals to human; compounds found to be P-gp substrates in rodents are likely to also be substrates in higher species but sufficient blood-brain barrier permeability may be retained in humans to allow the compound to act at intracerebral targets.

DMD #24745

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26

DMD #24745

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DMD #24745

Footnotes

DMD #24745

Figure Legends

Figure 1. Chemical structures of the three studied radiotracers. The asterisk represents the radiolabel position.

Figure 2. (A) Brain concentrations (SUV) and (B) brain-to-plasma concentration ratios (K_p) in monkeys (triangles), rats (circles) and guinea pigs (diamonds) after administration of $[^{11}C]$verapamil. Solid and broken lines represent average values in animals without and with additional CsA administration. Standard errors are shown as y-error bars if $n \geq$ 3. SUV and K_p were highest in monkeys, but the increase in brain concentrations after CsA treatment was greatest in rats.

Figure 3. (A) Brain concentrations (SUV) and (B) brain-to-plasma concentrations ratio (K_p) in humans (squares) (Syvänen, 2003), monkeys (triangles), rats (circles) and guinea pigs (diamonds) after administration of $[^{11}C]$GR205171. Solid and broken lines represent average values in animals without and with additional CsA administration. Standard errors are shown as y-error bars if $n \geq 3$. SUV and K_p were highest in monkeys and humans, but the increase in brain concentrations after CsA treatment was greatest in rats.

Figure 4. (A) Brain concentrations (SUV) and (B) brain-to-plasma concentration ratios (K_p) in humans (squares) (Liptrot et al., 2004), minipigs (triangles) and rats (circles) after administration of $[^{18}F]$altanserin. Solid and broken lines represent average values in animals without and with additional CsA administration. Standard errors are shown as y-

31

error bars if n ≥ 3. The brain concentrations were highest in minipigs, in which SUV, but not K_p, increased after CsA administration. Both SUV and K_p were increased in rats after CsA.

Figure 5. Sagittal PET images (summation 0-60 min) of [^{11}C]GR205171 in monkey, guinea pig and rat brain without (upper panel) and with (lower panel) CsA administration of CsA 15 mg/kg + 5 mg/kg/h (monkey) or 22.5 mg/kg + 7.5 mg/kg/h (guinea pig and rat) starting 20 min prior to a bolus injection of [^{11}C]verapamil.

Figure 6. Sagittal PET images (summation 0-60 min) of [^{11}C]verapamil in cynomolgus monkey brain without (upper panel) and with (lower panel) administration of CsA 15 mg/kg + 5 mg/kg/h starting 20 min prior to a bolus injection of [^{11}C]verapamil.

Figure 7. Sagittal PET images (summation 0-60 min) of [^{18}F]altanserin in pig brain without (upper panel) and with (lower panel) CsA administration of CsA 22.5 mg/kg + 7.5 mg/kg/h starting 20 min prior to a bolus injection of [^{18}F]altanserin.

DMD #24745

Table 1. Comparison of amino acid content in P-glycoprotein in human, chimpanzee, rhesus monkey, dog, rat, guinea pig and mouse.

P-gp protein[b]	Percent of amino acid homology[a]						
	Human	Chimp-anzee	Rhesus monkey	Dog	Guinea pig[c]	Rat	Mouse
Mouse	87	80	85	83	82	93	100
Rat	85	83	88	83	80	100	
Guinea Pig[c]	82	82	82	82	100		
Dog	87	89	87	100			
Rhesus monkey	93	94	100				
Chimpanzee	97	100					
Human	100						

[a]The percentage identity calculated using Ensembl 45 protein sequences (Hubbard et al., 2007) and Blast 2 sequence comparison (Tatusova and Madden, 1999).

[b]P-glycoprotein, transcribed from MDR1 (human, chimpanzee, rhesus monkey, dog, guinea pig) and mdr1a (rat and mouse) genes.

[c]The whole gene sequence is not yet available. The gene was predicted by projecting human Ensembl transcripts through a BLASTZ DNA alignment to the human genome.

DMD #24745

Table 2. Numbers of subjects for each study and species involved. Numbers in

parentheses denote the number of subjects that received cyclosporin A.

	Rat	Guinea pig	Minipig	Monkey	Human
[^{11}C]verapamil	6 (3)	6 (3)	-	2 (1)	-
[^{11}C]GR205171	9 (6)[a]	6 (3)	-	5 (1)	5[c]
[^{18}F]altanserin	9 (4)[b]	-	6 (1)	-	5[d]

[a]Two dose groups of CsA, [b]Ex-vivo studies, [c](Syvänen, 2003), [d](Liptrot et al., 2004)

Table 3. Average (standard deviation, n≥3) baseline SUV and K_p values over 60 min in cerebellum for the studied radiotracers.

	[¹¹C]verapamil		[¹¹C]GR205171		[¹⁸F]altanserin	
	SUV	*K_p*	*SUV*	*K_p*	*SUV*	*K_p*
Rat	0.21 (0.05)	1.13 (0.36)	0.32 (0.05)	3.45 (0.52)	0.32 (0.11)[c]	0.23 (0.10)[c]
Guinea pig	0.14 (0.02)	0.65 (0.02)	0.20 (0.05)	2.13 (0.83)	-	-
Minipig	-	-	-	-	1.20 (0.14)	2.08 (0.28)
Monkey	0.40[a]	4.61[a]	1.57 (0.36)	9.62 (3.93)	-	-
Human[b]	-	-	2.11 (0.84)[b]	29.8 (7.0)[b]	0.90 (0.22)[d]	1.04 (0.49)[d]

[a]One animal, [b](Syvänen, 2003), [c]Ex-vivo studies; values at 60 min after radiotracer administration, [d](Liptrot et al., 2004)

Table 4. Average $SUV_{increase}$ and $K_{p,increase}$ over 60 min in cerebellum, striatum and frontal cortex after CsA administration (ns=not significant, $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$, compared to baseline values)

	[^{11}C]verapamil		[^{11}C]GR205171		[^{18}F]altanserin	
	$SUV_{increase}$	$K_{p,increase}$	$SUV_{increase}$	$K_{p,increase}$	$SUV_{increase}$	$K_{p,increase}$
Cerebellum						
Rat	4.1[*]	5.8[**]	3.6[**]	3.5[**]	2.6[*c]	2.3[*c]
Rat low dose CsA	-	-	1.4[ns]	1.5[**]	-	-
Guinea pig	3.9[***]	2.9[**]	2.3[**]	2.0[ns]	-	-
Minipig[a]	-	-	-	-	1.8	0.8
Monkey[a]	4.3	3.6	2.1	2.5	-	-
High binding region[b]						
Rat	-	-	-	-	2.6[*c]	2.9[*c]
Minipig[a]	-	-	-	-	2.0	0.9
Monkey[a]	-	-	2.6	2.9	-	-

[a]A single animal received CsA

[b]High binding region: striatum for [^{11}C]GR205171and frontal cortex for [^{18}F]altanserin.

[c]Ex-vivo studies; values at 60 min after radiotracer administration

DMD #24745

Table 5. Average percentage of the tracer not bound to plasma proteins without (w/o)

and with (w) cyclosporin A (CsA) administration (standard deviation if n≥3).

	[11C]verapamil		[11C]GR205171		[18F]altanserin	
	w/o CsA	w CsA	w/o CsA	w CsA	w/o CsA	w CsA
Rat	5.5 (1.9)	6.2 (2.2)	10.5 (0.5)	13.0 (0.6)[b]	<1	<1
Guinea pig	3.5 (1.8)	3.6 (1.4)	9.7 (3.0)	13.9	-	-
Minipig	-	-	-	-	<1	<1
Monkey 1	-	-	11.3, 9.4[a]	9.6	-	-
Monkey 2	8.4, 8.5[a]	7.9	11.0	-	-	-
Human	-	-	14.9 (1.6)	-	<1	-

[a]two scans were performed in the same animal. The second value was determined in

plasma obtain 1-2 min before CsA administration.

[b]significantly (p<0.001) higher with CsA than without according to a one-tailed t-test.

Figure 1

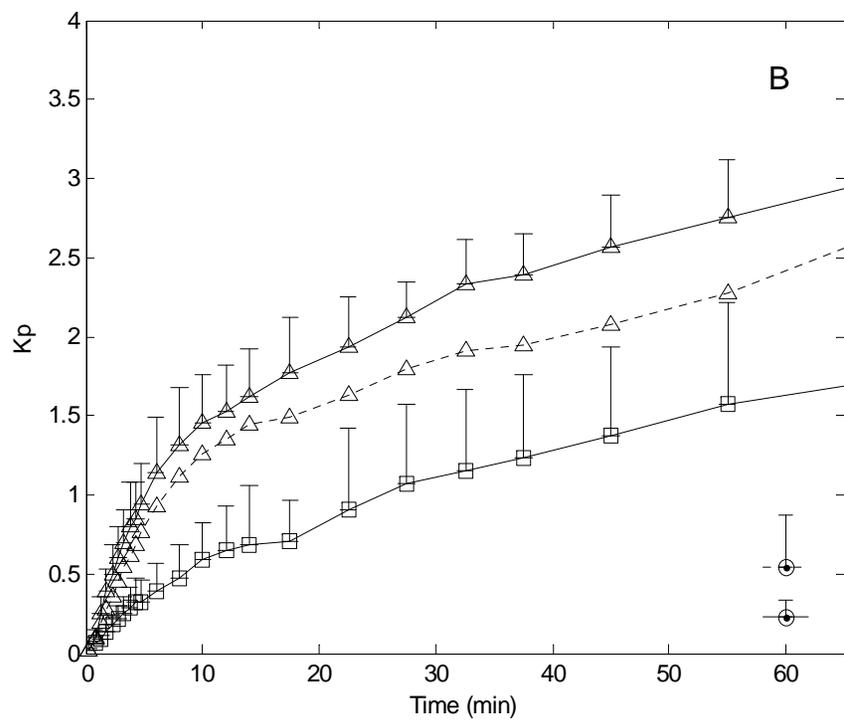
[¹¹C]verapamil
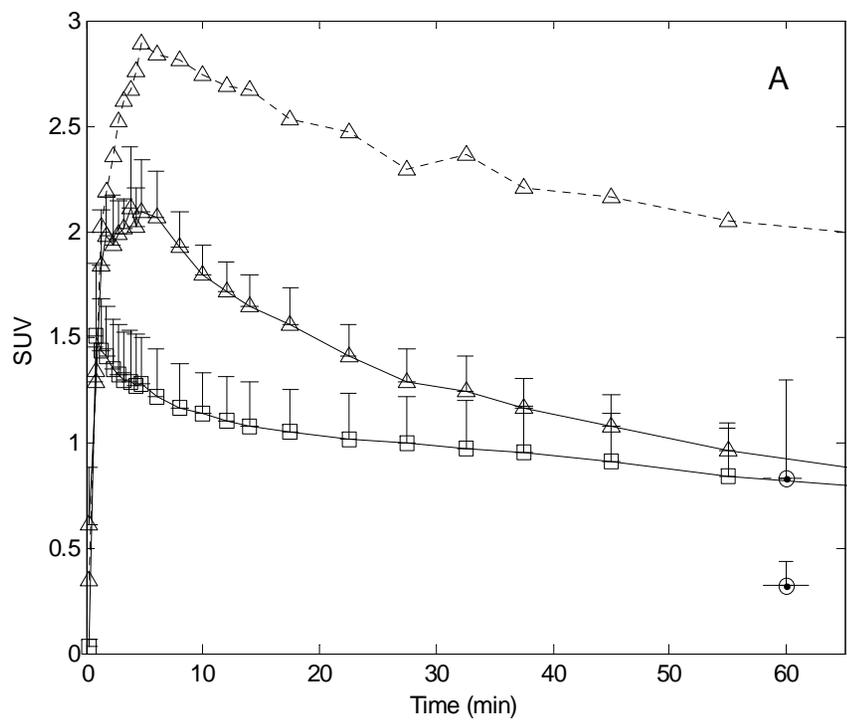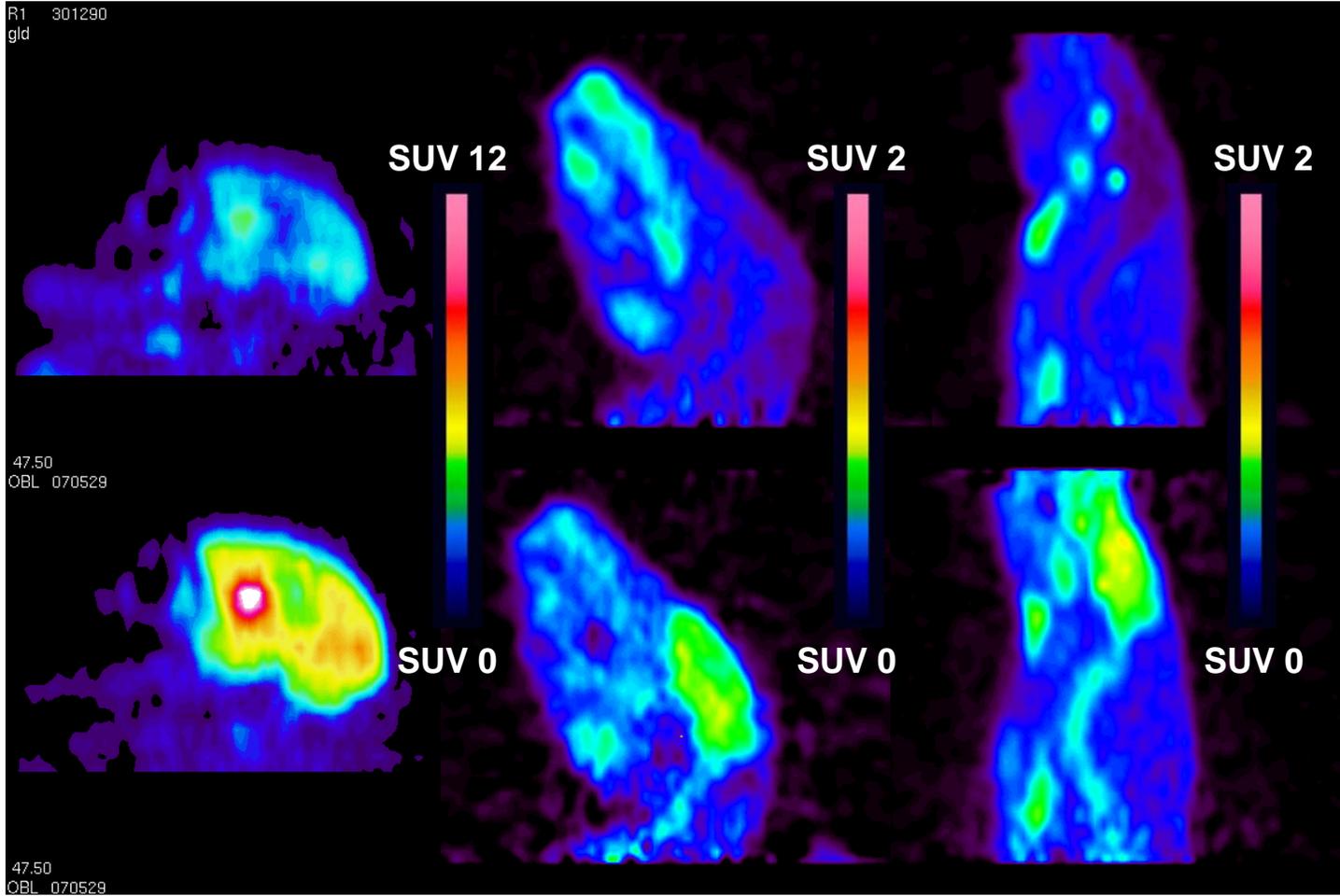
[¹¹C]GR205171

[¹⁸F]altanserin
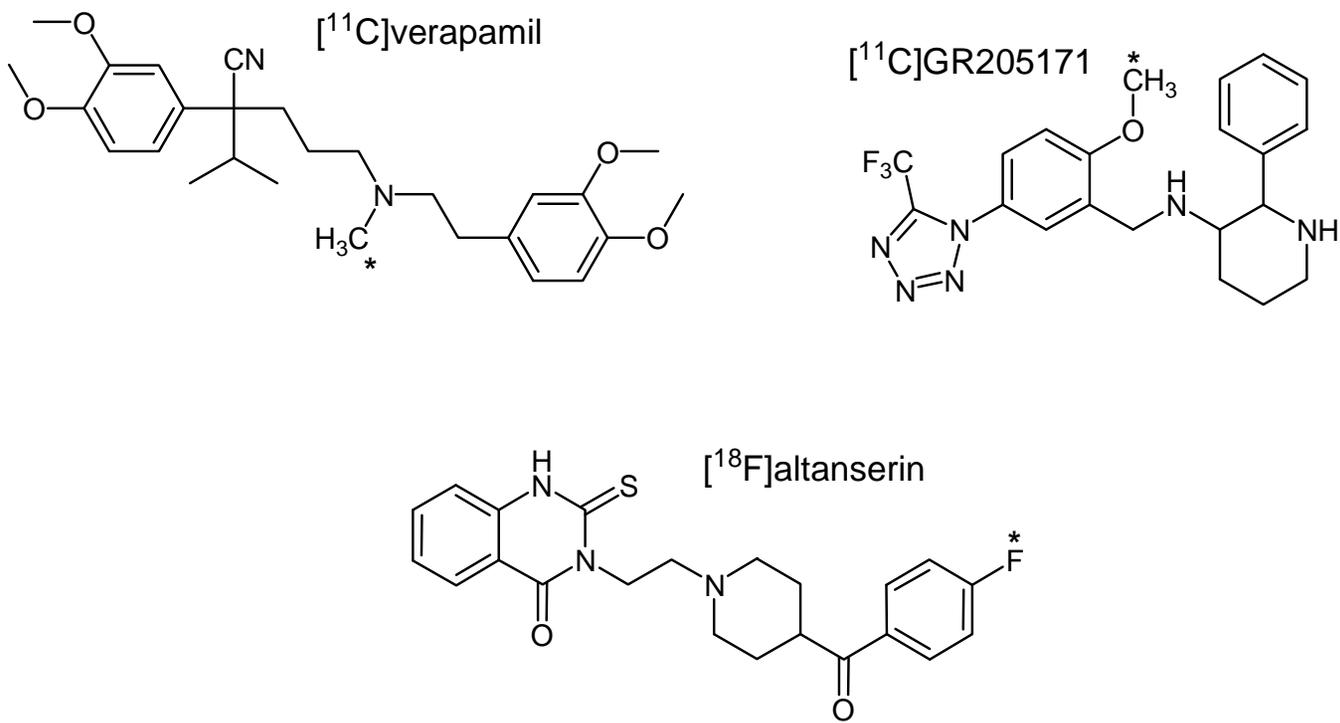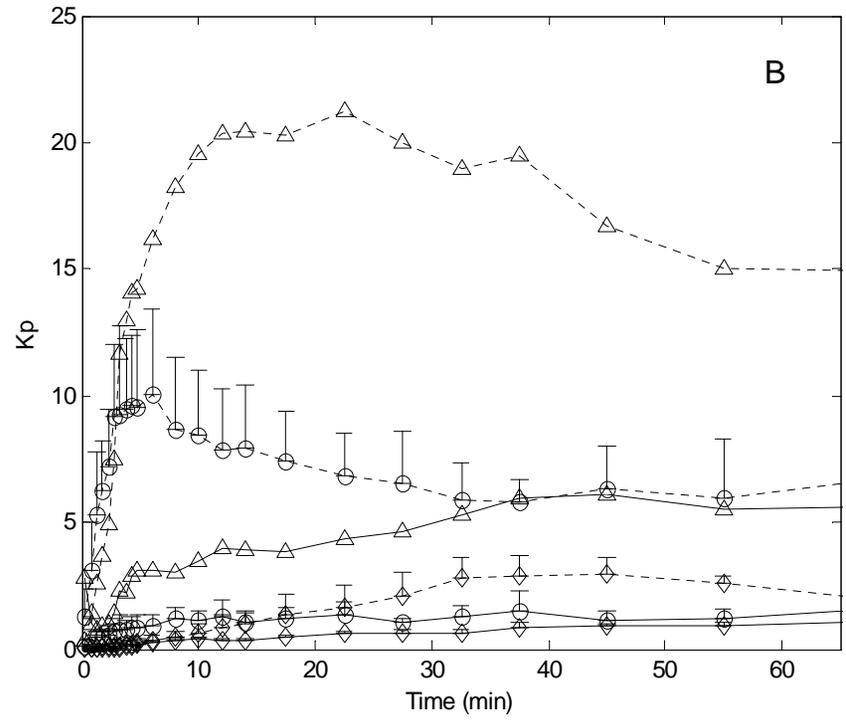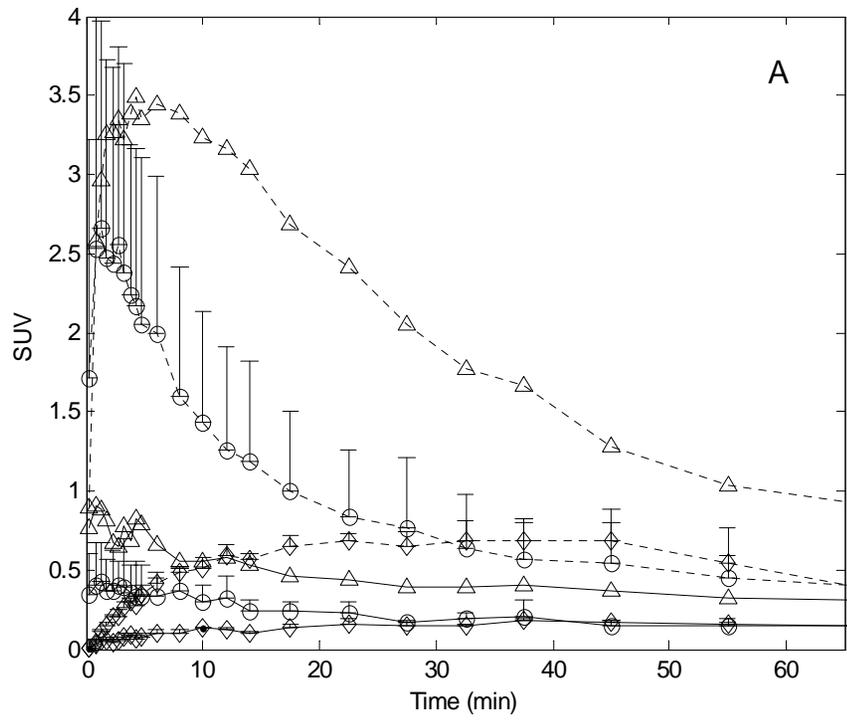
Figure 2

Figure 3

Figure 4

Figure 5

Monkey Guinea pig Rat

Figure 6

Figure 7